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Wounds increase activin in skin and a vasoactive neuropeptide in sensory ganglia

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Abstract

Successful healing of skin wounds requires sensory innervation and the release of vasoactive neuropeptides that dilate blood vessels and deliver serum proteins to the wound, and that cause pain that protects from further injury. Activin has been proposed as a target-derived regulator of sensory neuropeptides during development, but its role in the mature nervous system is unknown. While adult skin contains a low level of activin, protein levels in skin adjacent to a wound increase rapidly after an excision. Neurons containing the neuropeptide calcitonin gene-related peptide (CGRP) increased in sensory ganglia that projected to the wounded skin, but not in ganglia that projected to unwounded skin, suggesting that neurons respond to a local skin signal. Indeed, many adult sensory neurons respond with increased CGRP expression to the application of activin in vitro and utilize a smad-mediated signal transduction pathway in this response. A second skin-derived factor nerve growth factor (NGF) also increased in wounded skin and increased CGRP in cultured adult dorsal root ganglia (DRG) neurons but with lower efficacy. Together, these data support the hypothesis that activin made by skin cells regulates changes in sensory neuropeptides following skin injury, thereby promoting vasodilation and wound healing.

Keywords: Dorsal root ganglion; Sensory neuron; Activin; Transforming growth factor β; Bone morphogenetic protein; Calcitonin gene-related peptide; Target-derived; Smad; Skin wound

Introduction

Skin injury or inflammation results in both rapid local vasodilation caused by the sensory neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP), and longer-term upregulation of these neuropeptides in both skin and spinal ganglia. These neuropeptides are present in cutaneous sensory nerve terminals and are released centrally and peripherally during pain transduction (Holzer, 1988; Ju et al., 1987; Lee et al., 1985; Molander et al., 1987; O'Brien et al., 1989; Skofitsch and Jacobowitz, 1985). Multiple neuropeptides are normally present in small- and medium-sized sensory neurons that innervate skin, blood vessels, and gut, but are absent from larger-sized proprioceptive neurons that innervate muscle spindles. CGRP and SP are also released from sensory nerves in response to physical or chemical irritants, where CGRP causes peripheral vasodilation (Brain et al., 1985; Wallengren and Hakanson, 1987). Neuropeptide increases in the innervating dorsal root ganglia (DRG) occur following target tissue damage (Mulder et al., 1997), including arthritis (Hanesch et al., 1993). These increased neuropeptides are key biological responses, because neuropeptides and sensory innervation in general are required for successful wound healing (Kjartansson and Dalsgaard, 1987; Kjartansson et al., 1987). What is not well understood is how skin signals regulate sensory neuropeptides after a skin wound.

Previous work identified the TGFβ superfamily member activin as an important regulator of sensory vasoactive neuropeptide differentiation in embryonic sensory neurons (Ai et al., 1999; Hall et al., 2001), but the role of this ligand in adult neuropeptide regulation is not clear. The TGFβ superfamily comprises a large group of related ligands that have pleiomorphic effects in development (for review, see Hogan, 1996; Kingsley, 1994; Massagué and Chen, 2002; Mehler et al., 1997). Activin signals through a heteromeric receptor complex and activates the transcription factors smad2 and smad3. Once activated, these smads become

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phosphorylated and translocate to the nucleus to alter gene transcription (for review, see Heldin et al., 1997; Zhu and Burgess, 2001). Skin-conditioned medium or exogenous activin induces CGRP expression in embryonic sensory neurons (Ai et al., 1999; Hall et al., 2001) and activin is present in embryonic skin (Hall et al., 2002), suggesting that activin is a target-derived differentiation factor for developing sensory neurons. Activin mRNA is not detected in the skin of adult mice (Hübner et al., 1996), suggesting the ligand is not responsible for the maintenance of CGRP expression in adult DRG. By contrast, excisional skin wounds result in dramatic activin mRNA induction in adjacent skin (Hübner et al., 1996), which is thought to affect healing of the skin itself. The present study was designed to learn if such changes in skin activin following a wound affected not only local skin but also the innervating sensory neurons. Here we show that activin protein levels in adult skin increase following an excisional skin wound, that CGRP expression increases in the innervating sensory ganglia, and that adult neurons respond directly to activin stimulation in vitro via smad transcription factor activation. Nerve growth factor (NGF) can also affect sensory neuropeptide levels (Lindsay and Harmar, 1989), and we compared the expression and effects of activin and NGF. We propose that wounded skin upregulates activin in skin that signals cutaneous sensory neurons to increase CGRP, a change that results in increased vasodilation and delivery of serum proteins essential for successful wound healing.

Materials and methods

Skin wound and DRG analysis

Adult female Sprague–Dawley rats (8–10 weeks old, Zivic Miller) were deeply anesthetized by intraperitoneal injection (150 μl) of a cocktail of 100 mg/ml ketamine, 20 mg/ml xylazine, and 10 mg/ml acepromazine (obtained from the University Animal Resource Center). Each animal received eight 4-mm circular-square full thickness skin excisions along the L4 and L5 dermatomes in the left hind limb. For each excision, the full thickness of the skin, including the epidermis and dermis, was completely removed, leaving the fascia and muscle exposed. Schematic maps of rat dermatomes are based on antidromic C-fiber stimulation of dorsal root and extravasation of Evans Blue (unpublished observations, Dr. Carl Molander, Karolinska Institute, Stockholm). Following excision, wounds were left uncovered. Wounded animals moved, ate, and slept in a manner indistinguishable from unwounded animals. After 2 days, the 1–2 mm of skin surrounding the wounds, but not the scab, was collected. Skin from the unwounded hind limb as well as corresponding amounts of skin from unwounded animals served as controls. The fourth and fifth lumbar dorsal root ganglia (DRG) both contralateral and ipsilateral to the wound were collected. Ganglia were fixed for 2.5 h in 4% paraformaldehyde in 0.1 M PO_4, then cryoprotected overnight in 30% sucrose in PO_4 buffer at 4°C before sectioning. Three independent experiments were performed, with six animals in each of two experiments (two control/four wound) and eight animals in the third (three control/five wound). All experiments with animals were reviewed and carried out in accordance with the Institutional Animal Care and Use Committee at Case Western Reserve University.

Immunohistochemistry

Frozen sections (10 μm) from each DRG were collected on 10 sequential slides, such that adjacent sections on a slide were 100 μm apart, and stored at −20°C until use. DRG sections were washed in phosphate-buffered saline (PBS) and incubated for 1 h at room temperature in blocking solution (0.3% Tween 20, 0.2% Triton X-100, 3% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS). Sections were then incubated overnight at 4°C in primary antibody diluted in blocking solution. Antibodies included the neuronal nuclear marker mouse anti-Islet-1 (Avivi and Goldstein, 1999; 1:10, 39.4, Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-CGRP (1:4000, Sigma), or rabbit anti-Substance P (1:250, Incstar, Stillwater, MN). CGRP and SP signals were amplified by incubation with a biotin-conjugated anti-rabbit IgG diluted in blocking solution (1:300, Chemicon, Temecula, CA) for 1.5 h at room temperature, followed by incubation with a Cy2-conjugated Streptavidin diluted in PBS (1:800, Jackson Immunoresearch, West Grove, PA) for 1 h at room temperature. The primary antibody was omitted in control studies. Islet-1 was visualized by incubation with a Cy3-labeled anti-mouse IgG diluted in blocking solution (1:200, Jackson Immunoresearch). After washing, sections were mounted in 2% *n*-propyl gallate in 50% glycerol–PBS. All nuclei of cells that stained positive for Islet-1 were counted, and of these neurons, those that were CGRP positive were scored. Sections (10 μm) of 100 μm apart for both ipsilateral and contralateral DRG from five unwounded and nine wounded animals were assayed, with at least 1000 neurons counted for each DRG. Statistical analyses were done using Statview 4.1 software with an unpaired *t* test.

Adult skin was fresh frozen and 20-μm thick transverse frozen sections were collected and stored at −20°C until use. Sections were placed in −20°C acetone for 20 min, air dried for an additional 20 min, and incubated overnight at 4°C in primary antibodies: goat anti-activin (1:50 dilution, R&D Systems, Minneapolis, MN) and mouse pan-cytokeratin (1:100, Sigma) in dilution buffer that contained 0.3% Triton X-100–PBS and 2% BSA. After washing, sections were incubated in biotinylated donkey anti-goat IgG (1:200, Jackson Immunoresearch) and Cy2-conjugated goat anti-mouse IgG (1:200, Jackson Immunoresearch) in the same dilution buffer for 2 h at room temperature. Sections were washed and reacted with 1:750 dilution of Cy3-conjugated

streptavidin (Jackson Immunoresearch) for 1 h, then washed and mounted in 2% *n*-propyl gallate in 50% glycerol–PBS to prevent photobleaching.

Immunoblot lysates

Normal or wounded rat skin was homogenized on ice in 9-fold volume of lysis buffer containing 20 mM Tris, 0.1 M NaCl, 0.5 mM EDTA, 10% glycerol, 1% NP-40, one-tenth volume protease inhibitor cocktail (Sigma). For smad immunoblot lysates, DRG neurons were dissociated and plated as described below, except cells were cultured in 24-well plates with 8000 cells/1.88 cm^2. After 3 days in defined neurobasal medium alone (see below), 20 ng/ml activin was added for 1 h at 37°C. The cells were then lysed in 40 μl lysis buffer above. For all samples, insoluble material was removed by centrifugation, and the supernatant collected and stored at −20°C until use. Protein concentrations were determined by Peterson's modified assay (Sigma).

Western blot analysis

Total protein (10–15 μg) per lane in SDS-PAGE sample buffer (0.125 M Tris, 20% glycerol, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate, and 0.001% bromophenol blue) was boiled at 95°C for 5 min to reduce the proteins. The samples were run on a 15% 8 × 6 cm polyacrylamide gel under reducing conditions according to Laemmli (1970). Recombinant protein standards were used as positive controls (1 ng NGF, Austral Biologicals, San Ramon, CA; 2 ng human Activin A, R&D Systems). Proteins were transferred onto PVDF membrane (NEN, Boston, MA) for approximately 170 V-h. Nonspecific binding sites were blocked by incubation in 6% BSA in Tris-buffered saline (TBS) plus 0.2% Tween 20 for at least 6 h at 4°C. Membranes were incubated overnight at 4°C in appropriate primary antibody diluted in blocking solution: goat anti-recombinant human Activin A (1:400, R&D Systems) or rabbit anti-NGF (1:10,000, Sigma). As previously described, the activin antibody used in these experiments reacted only with the specified recombinant ligand (Hall et al., 2001). Primary antibody was omitted in control studies, and specific signal was absent. Following washes in TBS–Tween, membranes were incubated for 2 h at room temperature in appropriate peroxidase-conjugated secondary antibodies diluted in blocking solution: donkey anti-goat IgG (1:2000, Jackson Immunoresearch) or goat anti-rabbit IgG (1:2500, Jackson Immunoresearch). Membranes were washed and proteins visualized using a chemiluminescent substrate for horseradish peroxidase (SuperSignal West Pico, Pierce Chemicals, Rockford, IL) on Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

For Smad immunoblots, proteins from lysed DRG neurons were similarly separated by electrophoresis, but then transferred to nitrocellulose for approximately 170 V-h. Nitrocellulose was washed, then nonspecific sites were blocked for 30 min at room temperature in 5% nonfat dry milk, 0.05% Tween 20 in TBS before incubation overnight at 4°C in primary antibody diluted in blocking solution: rabbit anti-Smad2–PO_4 (1:400, United States Biological, Swampscott, MA). Following washes in deionized distilled water, the nitrocellulose was incubated 1.5 h at room temperature in appropriate peroxidase-conjugated secondary antibody: goat anti-rabbit peroxidase IgG (1:2500, Upstate Biotechnology, Lake Placid, NY, same diluent). Nitrocellulose was washed with water and then briefly washed with 0.05% Tween 20 in TBS before visualizing proteins using the chemiluminescent substrate as described above. In some cases, membranes were stripped in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCL for 30 min at 50°C. Membranes were washed, incubated in blocking solution for at least 2 h at room temperature, then incubated overnight at 4°C in goat anti-actin antibody (1:12,000, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation in peroxidase-linked secondary antibody (donkey anti-goat peroxidase, 1:2000, Jackson Immunoresearch), and washed and visualized as described above.

Adult DRG cell culture

Adult rat lumbar DRGs were collected on ice in Hank's Balanced Salt Solution (HBSS, Sigma), desheathed, and dissociated in dispase (2.5 u/ml, Roche Molecular Biochemicals, Indianapolis, IN)/collagenase (200 u/ml, Worthington Biochemical Corporation, Lakewood, NJ) in HBSS for 1 h 45 min at room temperature. Two thousand four hundred cells per 0.32 cm^2 were grown in 96-well plates coated with poly-L-lysine and 2 μg/ml laminin (Biomedical Technologies, Inc, Stoughton, MA) and maintained in defined neurobasal medium (Gibco-BRL) with B27 medium supplement (Gibco-BRL), penicillin–streptomycin (1:200, Gibco-BRL), 3 mM glutamine (unless otherwise specified). Cells were cultured for 3 days in defined neurobasal medium alone before addition of factors. Recombinant Activin A (1–20 ng/ml, R&D Systems) or NGF (1–100 ng/ml, Austral) was added for three additional days. In some cultures, FudR (5 μM, Sigma) was added to deplete non-neuronal cells.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h at room temperature, then fixed in ice-cold 95% methanol–5% glacial acetic acid for 15 min at −20°C. After rinsing in PBS, cultures were incubated in 0.4% Triton X-100 in PBS for 1 h at room temperature before incubation overnight at 4°C in primary antibody diluted in 0.4% Triton–PBS with 20% goat serum (rabbit anti-CGRP, 1:4000, Sigma; rabbit anti-SP, 1:250, Incstar). Following rinsing, cultures were incubated for 1.5 h at room temperature in biotin-conjugated goat anti-rabbit IgG (1:250, Chemicon). CGRP expression was visualized using

the ABC kit (Vector Laboratories, Burlingame, CA) with a diaminobenzidine chromogen. The percentage of total neurons that was CGRP positive was determined. Cells were scored based on intensity of staining (negative to light, medium, dark). A minimum of 250 cells per well was counted and only cells that scored medium to dark staining above background levels were included in the analysis. At least 3 wells per experiment were quantified for each condition from three independent experiments except the NGF dose response, done in duplicate. Statistical analyses were done using Statview 4.1 software with unpaired *t* tests. For smad2 translocation or phospho-smad2 immunocytochemistry, adult sensory neurons were dissociated and cultured as described above, except cells were grown on coverslips in 24-well plates at 8000 cells/1.88 cm^2. After 3 days, neurons were stimulated with 20 ng/ml activin for 1 h at 37°C. The defined medium alone served as control. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature, then in 95% methanol−5% acetic acid for 12 min at −20°C. After washing in PBS, cells were reacted with antibodies to either smad 2 (1:250; Upstate Biotechnology) or phospho-specific smad2 (Smad2−PO_4, 1:500; United States Biologicals) followed by biotin-conjugated secondary antibody (1:300, Chemicon) and then Cy2-conjugated streptavidin (1:800, Jackson Immunoresearch). Coverslips were then mounted in 2% *n*-propyl gallate in 50% glycerol−PBS. Smad2 translocation immunostaining was performed in one experiment, and smad2 P04 immunostaining was done in six independent experiments.

Results

Both sensory innervation and the release of vasoactive neuropeptides in the skin are crucial for successful skin wound healing. Previous work identified activin as an important regulator of sensory vasoactive neuropeptide differentiation in embryonic sensory neurons (Ai et al., 1999; Hall et al., 2001) and demonstrated that activin mRNA increased following skin wound (Hübner et al., 1996), yet the role of activin in adult neuropeptide regulation is not clear. The present study was designed to learn if changes in skin activin following a wound affected not only local skin but also the innervating sensory neurons. To examine activin and CGRP changes after skin wounds, full thickness excisional skin wounds were made in one hind limb of adult rats along the fourth and fifth lumbar dermatomes (Fig. 1). Two days later, the skin surrounding each wound was collected and analyzed for the presence of activin and NGF via Western blot. In addition, the lumbar DRGs that innervate the wounded skin region were analyzed for changes in vasoactive neuropeptides. Further, the ability of activin to increase CGRP in dissociated adult neurons was tested. Our results suggest that activin from a skin wound acts on innervating sensory neurons to increase neuropeptides.

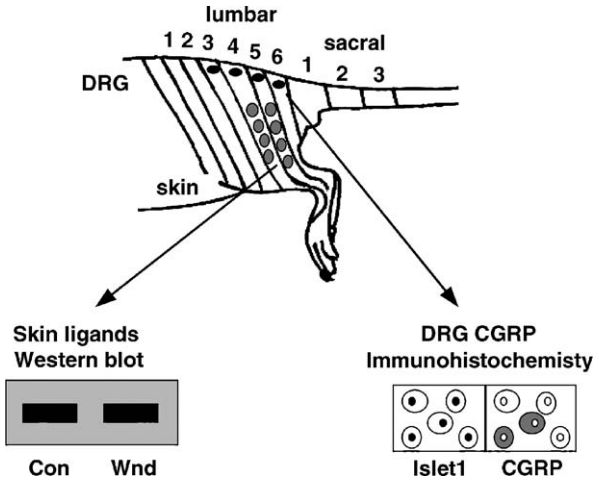

Fig. 1. Experimental paradigm. Four full thickness excisional skin wounds were made in both the L4 and L5 dermatomes of one hind limb of an adult rat. Two days later, the healing skin was collected for Western immunoblot, and the innervating DRG analyzed for the proportion of CGRP- and Islet-1-expressing neurons.

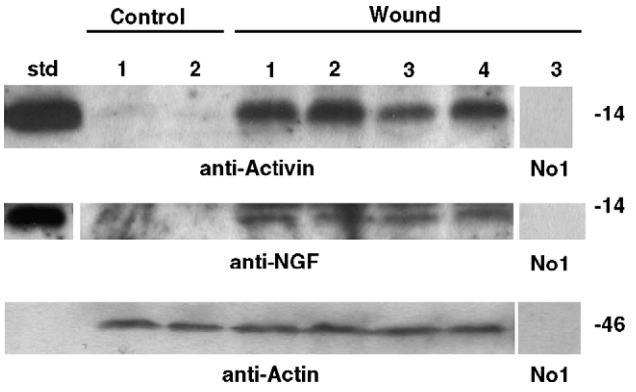

Fig. 2. Activin A and NGF proteins increase in skin following wound. Western blot analyses of skin lysates to identify activin A (top panel) or NGF (middle panel) in skin from each control or wounded animal. Recombinant activin A (2 ng, std lane) has Mr 14 kDa. Low levels of activin A are present in control unwounded skin samples (Control lanes) and after wound, activin A levels increased in each wounded animal (Wound lanes). Recombinant NGF (1 ng, std lane) appears at Mr 13 kDa. Low levels of NGF are present in unwounded skin. NGF protein levels increased following skin wound in each animal. When primary antibody was omitted, no reactivity was seen (No 1). As a loading control, the activin blot was stripped and reprobed for actin (lower panel) to demonstrate that similar amounts of protein were present. Similar results were seen in three independent experiments, with six animals in each of two experiments (two control/four wound) and eight animals in the third (three control/five wound).

Excisional skin wounds increased activin expression in surrounding, healing skin. Although activin A is present in embryonic skin (Hall et al., 2002), activin mRNA is difficult to detect in unperturbed adult skin (Hübner et al., 1996). Following a skin wound, the amount of activin protein increased in skin at 2 days (Fig. 2, top panel, wound lanes), but did not increased in unaffected skin on the opposite leg of the same animal or in unwounded animals. Activin is expressed in the epidermal keratinocytes of the skin at the

wound site but not in normal skin (Fig. 3). Because NGF can affect sensory neuropeptide levels and increases in skin following inflammation (Lindsay and Harmar, 1989; Malcangio et al., 1997; Woolf et al., 1994), unwounded and wounded skin were also analyzed for changes in NGF expression. As expected, very low levels of NGF were present in unwounded adult skin, and NGF also increased in adjacent skin following the wound (Fig. 2, middle panel). Thus, a skin wound induces local changes in expression of skin ligands.

To test if the skin wound affected sensory neuropeptides, the corresponding fourth and fifth lumbar DRGs on either the wounded side (ipsilateral) or on the opposite side (contralateral) of the animal were analyzed for CGRP and SP expression. DRG sections were reacted with antibodies to the transcription factor Islet-1 to identify the nuclei of all neurons (Avivi and Goldstein, 1999) and a CGRP antibody to identify the neuropeptide-containing sensory neurons that include nociceptors (Fig. 4A, arrows). As expected, Islet-1 antibody identified comparable proportions of DRG neurons in control and wounded animals. About one-fourth of DRG neurons were CGRP-positive in unwounded animals (Fig. 4B, L4 DRG 24.2 ± 1.45% (n = 7729); L5 DRG 24.2 ± 1.59% (n = 8312)), and both the right and left DRGs of unwounded animals contained similar proportions of CGRP-immunoreactive neurons, as expected (Fig. 4B, black bars). In contrast, the DRG ipsilateral to the wounded side demonstrated a 37.1 ± 5.1% increase in the proportion of CGRP-containing neurons in L4 ganglia and a 32.2 ± 7.4% increase in L5 ganglia (Fig. 4B, gray bars, P = 0.01 and P = 0.017, respectively). Further, because skin injury was performed on only one side of the animal, the proportion of CGRP-containing neurons was unchanged in the DRG opposite the wound, suggesting that the wound did not produce a systemic effect. Thus, the excisional skin wound increased CGRP expression in the neurons of the innervating DRG.

The increased activin in wounded skin and increased CGRP in corresponding DRG suggest that skin activin

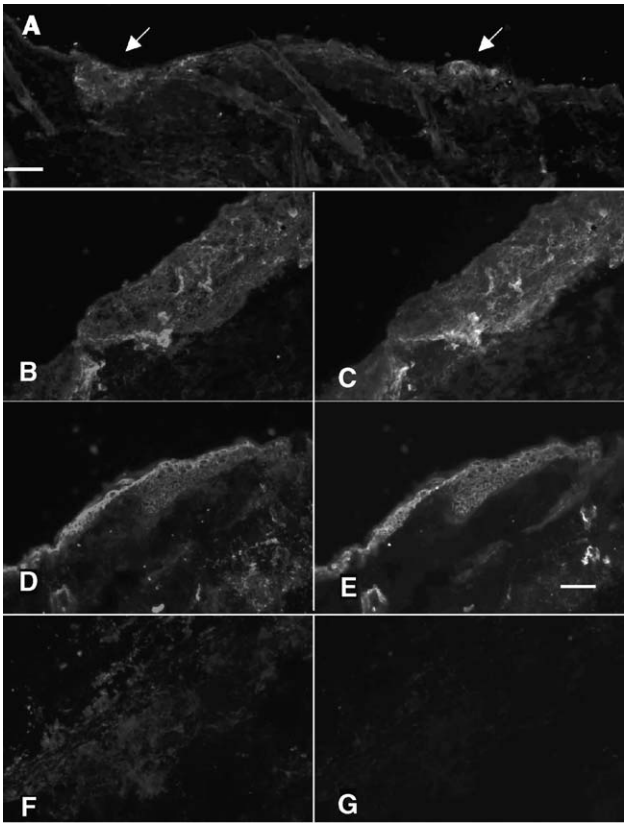

Fig. 3. Activin is expressed in keratinocytes. (A) Low magnification of skin 2 days following minor wounds caused by full thickness incisions (arrows). Scale bar: 100 μm. (B–G) Higher magnification of skin 2 days following large excisional wounds. (B and D) Anti-activin. (C and E) Anti-cytokeratin. While normal skin has light activin reactivity, activin is highly expressed in responding keratinocytes at the wound margin. These epidermal cells also react with anti-cytokeratin. (F and G) When primary antibody is omitted, no specific staining is seen at wound. Scale bar: 25 μm.

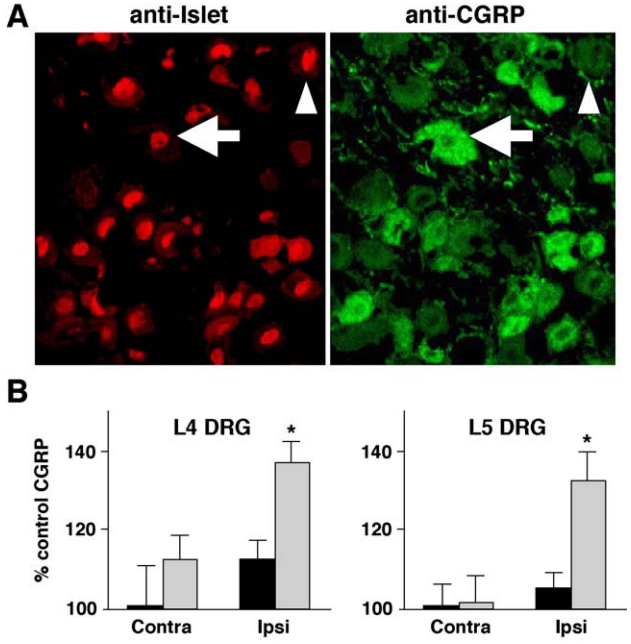

Fig. 4. CGRP-immunoreactive neurons increase in DRGs that innervate the skin wounds. (A) Only neurons with an Islet-1-immunoreactive nucleus were counted as neurons and evaluated for CGRP expression. CGRP-immunoreactive (arrows) and CGRP-deficient neurons (arrowheads) were identified and quantified as a percentage of total neuron number. (B) The percentage of CGRP-positive neurons in the L4 DRG of unwounded animals (black bars) was 24.2 ± 1.45 SEM in 7729 neurons counted from five animals, while in the L5 DRG of these animals 24.2 ± 1.59 SEM neurons were CGRP immunoreactive in 8312 neurons counted (black bars). The percentage of CGRP immunoreactive neurons was normalized to these control values, and in DRG ipsilateral to the wound, a 37.1 ± 5.1% increase in CGRP-containing neurons was observed in L4 ganglia and a 32.2 ± 7.4% increase observed in L5 ganglia (L4, 9020 neurons counted, P = 0.01; L5, 11,256 neurons counted, P = 0.017; nine wounded animals counted), while there was no change in DRG contralateral to the wound (L4, 9995 neurons counted, P = 0.23; L5, 10,517 neurons counted; P = 0.51).

modulates CGRP levels in adult neurons. To test this notion, adult DRG neurons were examined for their ability to respond to activin or NGF stimulation in vitro. Neurons were maintained for 3 days in defined medium to recover from axotomy, then stimulated with activin or NGF for an additional 3 days. In control cultures, about 30% of neurons maintained in defined medium had detectable CGRP expression (Fig. 5), a proportion similar to that seen in vivo (Scott, 1992). Activin stimulation increased the percentage of CGRP-expressing neurons in a dose-dependent manner, such that 39.5 ± 1.11% of neurons expressed CGRP with 20 ng/ml activin (Fig. 5C, $P < 0.0001$). The addition of NGF to adult neurons in vitro also induced CGRP expression in a dose-dependent manner (Fig. 5D), but substantially higher concentration of ligand was required to produce an increase (50 ng/ml NGF, 38.6 ± 1.04%, $P = 0.001$). Activin stimulation produced robust CGRP induction at lower concentrations, suggesting that although neurons are capable of responding to multiple stimuli in vitro, activin may be playing the dominant role in CGRP induction. In some cases, adult neurons were treated with 5 μM FudR during the culture period to deplete nonneuronal cells. These neurons also responded to activin in a dose-dependent manner (Fig. 5E). To test if activin treatment potentiated NGF effects, 20 ng/ml activin was added for 2 days, followed by 50 ng/ml NGF for 1–2 days, or factors were added in the reverse order, or simultaneously, and CGRP-expressing neurons quantified (Fig. 5F). Activin added in addition to NGF, together or in either order, had equivalent effects to the addition of activin alone, indicating that these factors act on the same responsive neuronal population.

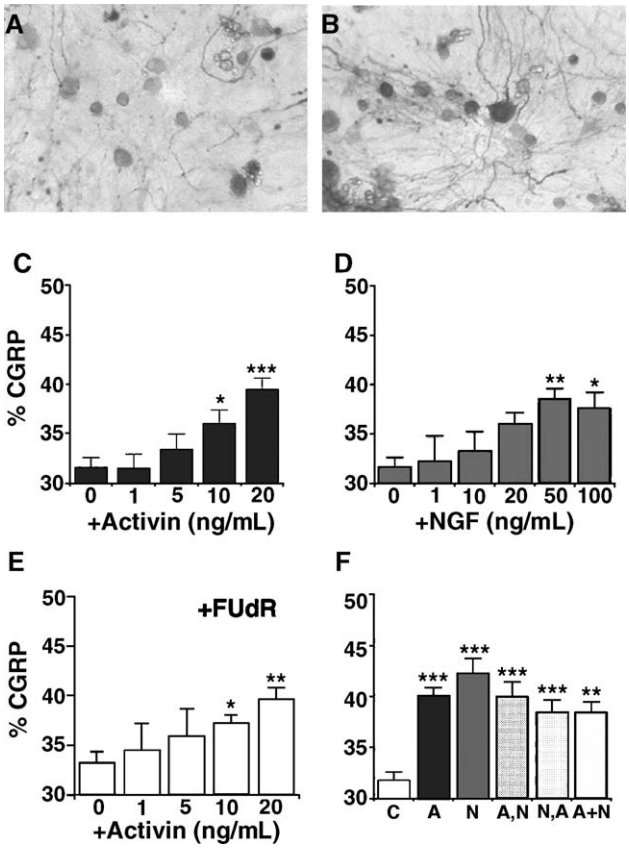

Fig. 5. Activin addition increases CGRP-immunoreactive neurons in adult DRG neurons in vitro. Approximately 30% of neurons cultured in basal medium alone were CGRP immunoreactive (A), while CGRP neurons increased after activin addition (B). Activin stimulated CGRP expression in adult DRG cultures in a concentration-dependent manner, with 20 ng/ml increasing CGRP neurons to 39.5 ± 1.11% of all neurons (C). The addition of 1–100 ng/ml NGF increased CGRP-immunoreactive adult DRG neurons to 38.6 ± 1.04%, with maximal induction seen at a concentration of 50 ng/ml NGF (D). In cultures treated with FudR to deplete nonneuronal cells, activin still increased CGRP-immunoreactive neurons (E, 39.6 ± 1.19%). The addition of 20 ng/ml activin before 50 ng/ml NGF, addition in reverse order, or addition of both factors simultaneously increased CGRP-immunoreactive neurons to levels equal to activin alone (F). Data represent means and SEM from three independent experiments done in triplicate, except NGF dose response, done in duplicate. *$P \leq 0.02$, **$P \leq 0.003$, ***$P \leq 0.0003$.

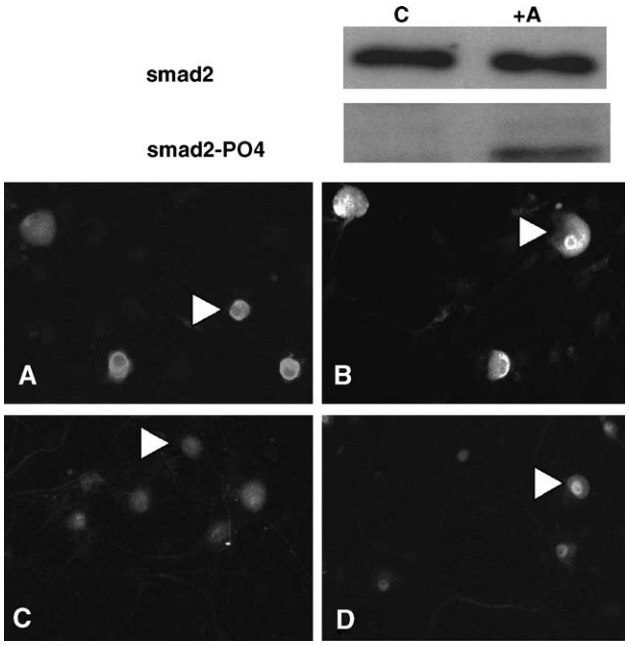

Fig. 6. Intracellular smad2 phosphorylation and translocation in neurons following activin stimulation. Dissociated adult DRGs were grown for 3 days in basal medium, and then stimulated for 1 h with 20 ng/ml activin. Cells were lysed and assayed for total smad 2 and phospho-specific smad 2 (top panel). Similar levels of total smad2 were present in treated (+A) and control (C) cultures, while activated phospho-specific smad2 was present only after activin stimulation (+A). To identify which cells responded to activin, cultures were stained with antibody to smad2 (A and B) or phospho-specific smad2 (C and D). Following 1-h activin stimulation, many neurons had smad2 translocation into the nucleus (arrowheads, A and B), and phospho-specific smad2–PO_4-immunoreactivity was localized in neuronal nuclei (C and D, arrowheads). Phospho-smad2 was present in 20.7 ± 5% neurons in control cultures and 91 ± 5% neurons after activin stimulation (200 cells counted in each of six experiments).

To assay if adult neurons respond directly to activin, the smad intracellular signaling cascade that mediates intracellular signals from activin was investigated in adult DRG neurons. In complete cultures, under basal conditions, there

was low smad2–PO_4 reactivity. Following 1-h activin treatment, there was strong induction of smad2–PO_4 detected on immunoblots (Fig. 6, upper panel). Total smad2 levels remained unchanged after stimulation, suggesting that existing pools of smad2 become activated in response to activin. These data indicate that a phosphorylated signal was generated after ligand binding, but the cellular location of the activated smad was unclear. The addition of activin for 1 h resulted in the translocation of smad2 from the cytoplasm to the nucleus in stimulated but not control cultures (Figs. 6A and B). Further, using antibody specific for phosphorylated smad2, few neurons had nuclear smad2–PO_4 (Fig. 6C) under basal conditions, while most had nuclear smad2–PO_4 after 1-h activin treatment (Fig. 6D, 20.7 ± 5% basal level; 91 ± 5% after activin stimulation, six experiments). Nonneuronal cells in these cultures did not show significant smad immunoreactivity or translocation, suggesting their trophic function observed by FudR studies above is not mediated by smads. Taken together, these data demonstrate that adult neurons alter their neuropeptide content in response to increases in activin that occur during the wound healing process.

Discussion

While skin wound healing might initially be considered a local event involving only intrinsic skin cells, successful healing actually requires sensory innervation and delivery of vasoactive neuropeptides to the skin (Holzer, 1988; Kjartansson and Dalsgaard, 1987; Kjartansson et al., 1987; Wallengren and Hakanson, 1987). The present study demonstrates that neuropeptide increases in DRG neurons occur after skin wounds as they do following inflammation (Mulder et al., 1997) and arthritis (Hanesch et al., 1993). Further, healing skin was shown to have increased levels of activin protein. Activin is capable of inducing adult DRG neurons to become CGRP-immunoreactive in vitro, supporting the notion that the receptors and signaling mechanisms required for these ligands to act are still present on many adult neurons. Indeed, smad transcription factor activation and translocation occur rapidly after activin addition in these cells. These novel observations highlight activin as a key regulator of neuropeptides in adult sensory neurons responding to a skin wound.

Several experimental paradigms are currently used to study sensory neurons after injury, and each manipulation is associated with distinct neuropeptide changes. The wound paradigm used in this study excises some target skin and is associated with increased CGRP expression in the innervating DRG. This increase in CGRP following a skin wound differs from the decreases in CGRP, SP, and somatostatin in the DRG after the nerve is cut (Villar et al., 1991; Zhang et al., 1996). Loss of target-derived factors such as NGF or presentation of nonneuronal cell-derived injury factors is thought to underlie the neuropeptide changes that follow axotomy (Fizgerald et al., 1985; Zigmond, 1997; Zigmond et al., 1995). A third manipulation, skin inflammation, involves injection of chemical irritants under the skin and is also associated with increased CGRP and SP in the innervating DRG (Neumann et al., 1996). Skin inflammation increases NGF expression in the periphery, and NGF increases can alter sensory neuropeptide content (Donnerer et al., 1992; Lindsay and Harmar, 1989; Woolf et al., 1994). However, systemic administration of anti-NGF reagents only partially blocks the increase in DRG neuropeptides after inflammation (Donnerer et al., 1992; Woolf et al., 1994). These data suggest that while NGF is involved in some neuropeptide changes, other molecules may play additional roles.

This is the first report of activin's ability to regulate CGRP expression in adult neurons. During embryogenesis, we propose that activin is a skin-derived differentiation factor (Hall et al., 2001, 2002). Indeed, expression of activin and other TGFβ family members is widespread in embryonic target tissues and may be focused by co-expression of inhibitory factors or additional growth factors. For example, BMPs and neurotrophic factors exhibit some synergism in survival-promoting activity in sensory neurons (Farkas et al., 1999). Although induction of CGRP expression in developing sensory neurons involves target-derived activin, low levels of activin in normal adult skin suggest that activin does not maintain neuropeptide expression (this report, Hübner et al., 1996). Increased activin in wounded adult skin may in part recapitulate the inductive role activin has on CGRP expression in sensory neurons. Unlike embryonic neurons, adult DRG neurons have already made functional connections with their target tissues, and about 30% of these neurons normally express CGRP in vivo (Scott, 1992). Similarly, one-third of adult neurons maintained in defined medium were CGRP-immunoreactive under control conditions, while 40% expressed CGRP after addition of activin. Adult neurons responded to activin in a dose-dependent manner and rapidly activated smad transcription factors upon ligand addition, indicating that activin acts directly on these neurons, and that some adult neurons continue to exhibit phenotypic plasticity. A comparison of activin's effects on embryonic and adult DRG neurons indicates that CGRP induction in adult neurons required higher concentrations of ligand and fewer neurons were capable of acquiring CGRP expression. Pilot studies suggest that 50 ng/ml activin is less effective at CGRP induction in adult neurons than the 20 ng/ml used in this study (B.A.C. and A.K.H., unpublished observations), suggesting a "plateau" in responsiveness occurs at these concentrations. Many small-diameter neurons were CGRP-immunoreactive in adult cultures, as expected because this neuropeptide is one hallmark of the nociceptor population. The majority of CGRP-expressing nociceptors also express substance P. Interestingly, the proportion of SP-containing neurons did not change after a skin wound (data not shown), suggesting that neuropeptides may be differentially regulated in response to a skin wound. Further, the

magnitude of CGRP induction in vitro was larger than that in vivo, perhaps reflecting the concentration or availability of the ligand after the skin wound. No difference was seen in the survival of adult rat DRG neurons that are NGF independent for survival even at high concentrations of activin, or with the addition of both factors together, thus confirming that activin's action was on CGRP induction, not selective survival. Although they do not require NGF for survival, adult sensory neurons still respond to NGF with limited neuropeptide increases (this report, Lindsay and Harmar, 1989; Lindsay et al., 1989). The original identity of these plastic neurons has not been fully established and may differ in vivo and in vitro. For example, only cutaneous sensory neurons are likely to be affected by skin changes in vivo, while the entire sensory population is exposed to activin in vitro.

While both activin and NGF can increase sensory neuropeptides (Ai et al., 1999), these ligands may have different functions following skin wounds. Previous studies have shown that activin mRNA increases in skin 1 day after skin injury and remains elevated for 7 days after wounding (Hübner et al., 1996), and our data indicate that activin protein is similarly upregulated after wounding in keratinocytes. Activin mRNA has also been detected in mesenchymal cells of the granulation tissue under a scab, as well as in activated macrophages (Erämaa et al., 1992; Hübner et al., 1996). In transgenic mice, overexpression of activin from a keratin promoter produces a thickened epidermis resulting from hyperproliferation of keratinocytes (Munz et al., 1999). Excisional skin injury in these mice resulted in enhanced granulation tissue formation and enhanced wound healing. In addition, mice that overexpress the activin antagonist follistatin have significantly delayed wound healing (Wankell et al., 2001). Several other members of the TGFβ superfamily stimulate keratinocyte or fibroblast proliferation and other aspects of wound repair (see Choi and Fuchs, 1990; Hebda, 1988; Roberts and Sporn, 1986), but these have varied expression in wounded skin. Activin appears to play a key role in wound repair of adult skin, and although it is possible that changes in untested members of the TGFβ family could contribute to the observed peptide changes, increases in skin activin following a wound, along with its ability to increase neuronal CGRP, make activin a prime candidate for a role in neuropeptide regulation. A more important role for NGF after injury may be in the regulation of cutaneous afferent sprouting (Rajan et al., 2003). Administration of anti-NGF at the site of injury reduces sprouting locally and prevents hyperalgesia, suggesting NGF acts in pain responses after injury (Ramer and Bisby, 1999; Ro et al., 1999; Streppel et al., 2002).

It is reasonable to assume that the activin signal in skin is transmitted to the neuronal cell body and subsequently results in CGRP induction, although little is known about the molecular basis of this signal in neurons. Other target-derived factors such as NGF generate a retrograde signal in neurons (Ginty and Segal, 2002) that is thought to reflect a signaling endosome, but whether activin, its receptors, or associated smad cell signaling moieties are retrogradely transported is not currently known. Recent pilot data suggest that radiolabeled BMP7 can be retrogradely transported in sympathetic neurons (Beck et al., 2001), and BMP4 appears to be retrogradely transported from targets in invertebrate neurons (Allan et al., 2003). Other members of the TGFβ family are internalized along with their receptors in cultured cell lines to signal to the nucleus (Panopoulou et al., 2002; Zwaagstra et al., 2001), but molecular details of activin signaling from axons remain to be elucidated.

We have identified activin as a new candidate regulator of sensory vasoactive neuropeptide regulation that induces CGRP in embryonic neurons and that is strongly upregulated after skin injury in adult sensory neurons. We propose that during development, limiting amounts of activin in skin induce a subset of cutaneous sensory neurons to express CGRP, and this subset of neurons is maintained in the adult. After adult skin injury, the initial release of vasoactive sensory neuropeptides at the injury site produces rapid vasodilation and release of serum factors that are important for wound healing. It is interesting to speculate that the mechanism by which target-derived activin induces CGRP during development may be recapitulated in the adult after skin injury. These molecules may then trigger activin expression in skin or blood vessels, which, perhaps in combination with NGF, induces sensory neurons to increase their production of vasoactive peptides (feed-forward) and to regulate wound healing. Thus, to successfully understand sensory neuronal responses to injury that involve pain and promote healing, it is crucial to understand the functions of the TGFβ family mediating these biological actions.

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